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REVIEW

J.K. Mfopou · L. Bouwens

Hedgehog signals in pancreatic differentiation from embryonic stem cells: revisiting the neglected

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Abstract Recent demonstrations of insulin expression by progenies of mouse and human embryonic stem (ES) cells have attracted interest in setting up these cells as alternative sources of β-cells needed in diabetes cell therapy. It is widely acknowledged that information gathered in the field of developmental biology as applied to the pancreas is of relevance for designing *in vitro* differentiation strategies. However, looking back at the protocols used so far, it appears that the natural route toward the pancreas, which goes via the definitive endoderm, was usually bypassed. As a consequence Hedgehog signaling, the earliest inhibitor of pancreas initiation from the endoderm, was generally not considered. A recall of the status of this pathway during ES cell differentiation appears necessary, especially in the light of findings that Activin A treatment of mouse and human ES cells coax them into definitive endoderm, a lineage showing wide Hedgehog ligands expression with the potential to hinder pancreatic programming.

Key words Activin · Hedgehog · definitive endoderm · diabetes · embryonic stem cells · pancreas development

Introduction

Diabetes mellitus is a leading endocrine disease with increasing prevalence and morbidity. Recent success in the transplantation of pancreatic islets of Langerhans indicates that diabetes can be controlled long term without any requirement for exogenous insulin injections. Because of the severe shortage in islet donors, this therapeutic option is still far from being generalized to all patients in need of transplantation. This situation stimulated the search for alternative sources of β-cells, including embryonic stem (ES) cells (Kaczorowski et al., 2002; Street et al., 2004; Bonner-Weir and Weir, 2005; Nir and Dor, 2005).

ES cells are pluripotent cells derived from the inner cell mass of the early blastocyst, with the potential to differentiate into derivatives of ectoderm, endoderm, and mesoderm. They are therefore regarded as a possible source of β-cells. This theoretical application of ES cell research will become true, provided pure clinical-grade β-like cells can be efficiently and reproducibly generated *in vitro*. Several studies showed expression of pancreatic genes, notably insulin, in derivatives of mouse and human ES cells following various differentiation protocols, including modulation of the culture microenvironment or overexpression of key transcription factors (Soria et al., 2000; Assady et al., 2001; Lumelsky et al., 2001; Hori et al., 2002; Shiroi et al., 2002; Kahan et al., 2003; Kim et al., 2003; Moritoh et al., 2003; Blyszczuk et al., 2004; Ku et al., 2004; Segev et al., 2004; Bai et al., 2005; Shi et al., 2005; Lavon et al., 2006; Vaca et al., 2006). Some studies claimed normalization of glycemia in transplanted diabetic animals, and raised hopes for a "diabetes cure" using ES cell derivatives. However, these protocols were generally not straightforward; they were also neither highly efficient in terms of β-cell number generated nor consistently reproducible between experiments or groups. Furthermore, they were not usually based on first generating definitive endoderm from which the pancreas develops *in vivo*. As such, it is doubtful whether insulin-positive cells detected among ES cell progenies following these strategies represent true β-cells or lineages

J.K. Mfopou · L. Bouwens (✉)
Cell Differentiation Unit
Diabetes Research Center
Faculty of Medicine and Pharmacy
Vrije Universiteit Brussel
Laarbeeklaan 103
1090 Brussels, Belgium
Tel: +32 2 477 4457
Fax: +32 2 477 4405
E-mail: lucbo@vub.ac.be

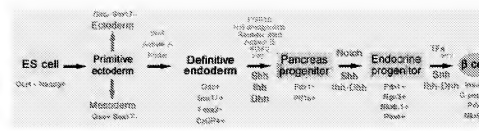


Fig. 1 A model for multi-step β-cell derivation from embryonic stem cells. High level of transforming growth factor β activity increases the proportion of definitive endoderm while reducing other fates. This endoderm is characterized by Gsc, Sox17, Foxa2, and CxCR4 expression. Soluble factors needed to convert endoderm cells into Pdx1+/Ptf1a+pancreas progenitors are still elusive, but Hedgehog signaling is well established as an inhibitor of this transition, as well as further differentiation into mature exocrine and endocrine cells. Expression of Ihh/Dhh in pancreatic epithelium at later stages of development and in adult islet suggests a role for a basal pathway activity. Color codes: brown, germ layers not wanted; blue, cell identity markers; green, positive influence; red, negative influence; ???, factors not yet unraveled.

reminiscent of developmental pathways in fetal liver, yolk sac and neuronal tissues that generate marginal cells expressing the non-pancreas specific insulin II gene in mice (Rajagopal et al., 2003; Hansson et al., 2004; Sipione et al., 2004; Stoffel et al., 2004; Bonner-Weir and Weir, 2005). These controversies motivated novel trials that consider normal features of pancreas development (Fig. 1), and considerable achievements have been made in the derivation of definitive endoderm from ES cells, a germ layer from which the pancreas develops *in vivo* (Kubo et al., 2004; D'Amour et al., 2005; Tada et al., 2005; Yasunaga et al., 2005). Just recently, a study with human ES cells demonstrated in a five-step differentiation process that definitive endoderm cells can be induced up to 80% purity by Activin A and Wnt3a treatment, and that these cells can be coaxed toward pancreatic insulin-producing cells via *in vitro* equivalents of primitive gut tube, posterior foregut, and pancreatic precursors (D'Amour et al., 2006). This study therefore acknowledges the hypothesis that *in vitro* implementation of a basic knowledge of pancreas development acquired from developmental biology is critical for the differentiation of β-cells from ES cells. With support from our recently acquired data, this review will focus on the role that Hedgehog signals, an established inhibitor of pancreas development neglected in many studies, play in the differentiation of ES-derived endoderm cells toward pancreatic insulin-producing cells.

Evidence for Hedgehog activity in early pancreas development

The pancreas develops from two buds (ventral and dorsal) in the foregut endoderm around E8.5 in mice. The gut endoderm derives exclusively from the definitive endoderm as opposed to the visceral endoderm with which it shares several markers including E-cadherin, Sox17, and Foxa2 (Kubo et al., 2004; D'Amour et al., 2005; Yasunaga et al., 2005). These buds arise from presumptive pancreatic anlagen characterized by high Pdx1 expression and the absence of Shh, and repression of Shh within these regions is mediated by notochord-derived Activin B and Fgf2 (Apelqvist et al., 1997; Kim et al., 1997; Hebrok et al., 1998). The absence of Hedgehog expression from the pancreatic anlagen is striking, as components of this pathway are widely expressed by nearly all epithelial cells of the oral ectoderm and the entire gut endoderm, except at the level of Rathke's pouch (pituitary primordium) and pancreas (Apelqvist et al., 1997; Hebrok et al., 1998, 2000; Treier et al., 2001). In the ventral endoderm, this Shh repression is mandatory for pancreas initiation to occur by default, or else hepatic progenitors are established. Indeed, as little as 50 ng/ml of recombinant Shh peptide diverts isolated ventral endoderm from pancreatic fate, whereas inhibition of the pathway in the foregut endoderm by Cyclopamine treatment can extend the pancreatic anlagen up to the stomach and duodenum (Kim and Melton, 1998; Deutsch et al., 2001). These data indicate that Hedgehog signals are inhibitory for pancreas development as from the early stages of pancreas initiation from the gut endoderm.

Hedgehog pathway activation in the pancreas

In mammals, three Hedgehog ligands (Sonic Hedgehog, Shh; Indian Hedgehog, Ihh; and Desert Hedgehog, Dhh) have been identified that bind to the same receptors Patched (Ptc1, Ptc2) with similar affinity. In the absence of Hedgehog ligands, Patched protein inhibits the transmembrane protein Smoothened (Smo) and therefore the transcriptional activity of Hedgehog effectors (Gli1, -2, -3 in mammals). These inhibitions are released by Hedgehog binding to Patched, which initiates translocation of Gli1 to the nucleus and activation of target genes including Gli1 and Ptc1 (Echelard et al., 1993; Ingham and McMahon, 2001). To this order, Gli1

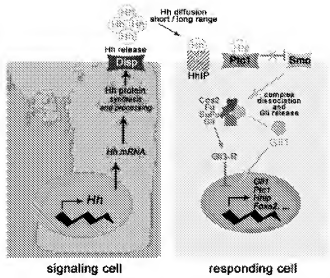


Fig. 2 The Hedgehog pathway. (**A**) The signaling cell synthesizes Hedgehog (Hh) precursors (Shh, Ihh, Dhh), which are cleaved autocatalytically to the N-terminus and C-terminus (autoprocessing machinery) fragments. The active N-terminus fragment is modified by cholesterol addition to its C-terminal end and fatty acyl modification of the N-terminal end (usually palmitoylation or myristoylation) before its release via the membrane protein Dispatched (Disp). These modifications ensure multimeric formation and increased potency, as well as Hh diffusion at short or long range to establish its function as a morphogen. (**B**) In the absence of Hh ligands (red labels and lines), the membrane protein Patched (Ptc1) inhibits the membrane localization of Smoothened (Smo), allowing for the formation of an inhibitory complex of Costal (Cos2), Fused (Fu), Suppressor of Fused (SuFu), and Gli. This complex favors the production of Gli repressor forms (Gli3-R), which inhibit transcription of Hh target genes. (**C**) In the presence of Hh ligands (green labels and arrows), Smo inhibition is released upon Hh binding to Ptc1. Smo translocates to the membrane and initiates the dissociation of the complex leading to Gli1 release. Gli1 can then enter the nucleus to activate target genes among which Gli1 itself. The responding cell can control the level of signaling by sequestering Hh ligands with the membrane protein Hedgehog-interacting protein (HhIP).

mRNA changes appear as the best readout of Hedgehog signaling (Fig. 2).

As previously mentioned, Pdx1 is the transcription factor that marks the domain of pancreas development in the gut endoderm. The promoter sequence that drives Pdx1 expression in the pancreatic anlagen can therefore be linked to the sequence of another protein to force its expression in the early pancreatic epithelium. Transgenic animal studies have extensively recognized the inhibitory function of Shh in early pancreas development. Expression of Shh under Pdx1 promoter leads to differentiation of intestinal mesenchyme from pancreatic mesoderm and severe alterations of pancreatic endocrine and exocrine cell differentiation (Apelqvist et al., 1997). Another study induced Shh in the pancreatic endoderm by mutation of the pathway antagonist Ptc1, ensuing constitutive activity by Smo function. This resulted in a complete absence of Pdx1+ and Glucagon+ cells in the presumptive pancreatic region by E9.5 in *Ptc1* $-/-$ mutants (Hebrok et al., 2000). Hedgehog interacting protein (Hhip) is expressed by target cells and plays a role in the modulation of the signaling level by binding the ligands and limiting their access to the receptors. It is therefore an antagonist of the Hedgehog pathway. Similarly, Hedgehog activity induced by homozygous mutation of Hedgehog interacting protein (*Hhip* $-/-$) results in disrupted pancreas differentiation, associated with lower levels of Fgf10 expression in the pancreatic mesenchyme (Kawahira et al., 2003). Ectopic expression of Shh and subsequent disruption of pancreas development is also identified in the presumptive pancreas region of mice bearing mutations in the Activin receptor IIB (*ActRIIB* $-/-$), further supporting the findings that notochord-derived Activin B suppresses Shh expression in the pancreatic region (Hebrok et al., 1998; Kim et al., 2000). These studies clearly demonstrate a negative role for Hedgehog signaling on pancreas development, in contrast to the vast majority of other vertebrate organs that are under its positive influence (Ingham and McMahon, 2001).

Hedgehog pathway inactivation in the pancreas

In order to confirm its inhibitory role, it is logical to predict that removal of all sources of Hedgehog signals would not alter pancreas development, but would rather result in enlarged organ size. In fact, double transgenic mice bearing mutations in *Shh* and *Ihh* loci (*Shh*$+/-$ *Ihh*$-/-$, *Shh*$-/-$ *Ihh*$+/-$) display pancreas sizes comparable to that of wild-type animals, whereas their body weight is severely reduced. Consequently, the relative pancreas size is increased in these animals, confirming the repressive function of the pathway (Hebrok et al., 2000). Although sufficiently convincing, these studies do not inform on the effect of complete Hedgehog absence as double null transgenic mice (*Shh*$-/-$ *Ihh*$-/-$) die at the early somite stage, before pancreas differentiation can be examined. However, they show evidence for Hedgehog dosage in the pancreatic endoderm given that in the complete absence of one Hedgehog homolog (*Shh*$-/-$ or *Ihh*$-/-$), reducing one allele of *Ihh* (*Shh*$-/-$ *Ihh*$+/-$) or *Shh* (*Ihh*$-/-$ *Shh*$+/-$) does not worsen the phenotype observed in homozygous mutants (Hebrok et al., 2000; Ramalho-Santos et al., 2000; Hebrok, 2003). The use of Cyclopamine, a plant alkaloid that potently inhibits Hedgehog pathway by interfering with Smo, also promoted pancreas development, and induced ectopic pancreatic tissue at stomach and intestine sites where low levels of the key pancreas transcription factor Pdx1 are normally expressed (Kim and Melton, 1998).

Evidence for Hedgehog activity in late pancreas development and in adult islets

The numerous studies mentioned in the previous sections underscore the inhibitory role of Hedgehog in the initiation of a pancreas program from the gut endoderm and its early stages of development, a function that additionally sets organ boundaries. The question remained whether this function persists as the pancreas develops further. To examine this outcome, transgenic mice bearing *Shh* or *Ihh* under *Pax4* promoter were generated, allowing their expression in the pancreas epithelium around E13 when *Pax4* is expressed in early endocrine progenitors. These animals displayed a severe reduction in pancreas mass, a defect in pancreatic epithelial cell expansion resulting in 90% loss of acinar cells and 80% loss of islet cells, and the remaining ducts appeared dilated (Kawahira et al., 2005). *Shh* was also induced *ex vivo* in E12.5 pancreas explants by Activin A but not by Activin B, resulting in the differentiation of intestinal muscle tissue with peristaltic activity. This latter study found no deleterious effect of Activin A treatment, and implicitly of Shh, on exocrine and endocrine differentiation within the explants (van Eyll et al., 2004). As the level of Shh protein expression was not evaluated in this study, it is not excluded that concentrations achieved were below the threshold required to affect pancreatic differentiation at this stage of development. A recent and elegant work demonstrated that *in vivo* overexpression of active β-catenin in E10.5–E11.5 mice pancreas ectopically induced Shh and Ptc1 within the epithelium, which was followed by loss of Pdx1+ progenitor cells and pancreatic hypoplasia. These data strengthened the previous findings that transgenic mice expressing Wnt1 or Wnt5a under *Pdx1* promotor show severe alterations of pancreas morphogenesis (Heller et al., 2002; Heiser et al., 2006). However, overexpression of β-catenin at later stages (E13.5) led to increased pancreas size. It remains unknown as to whether β-catenin also activated the Hedgehog pathway in developing pancreas when induced at this stage. That both Activin and β-catenin induce Shh expression in the pancreas requires further examination to determine whether the effect is direct or indirect. A crosstalk between these pathways has been established in other systems, indicating a positive or negative effect according to context. For instance, Activin positively regulates Wnt signaling (Wnt3a induction and sFRP1 repression) in human ES cells (Xiao et al., 2006); the Hedgehog targets Gli2/3 are regulated by Wnt signaling during avian somite cell specification (Borycki et al., 2000) whereas sFRP1 expression and β-catenin translocation are induced by Gli1 in 293 cells and in epidermal cells, respectively (He et al., 2006; Li et al., 2007). On the other hand, Indian Hedgehog appears to inhibit Wnt signaling during colon epithelial cell differentiation and Wnt5a overexpression reduces Shh signals in the lung epithelium (van den Brink et al., 2004; Li et al., 2005a). Nonetheless, all the data discussed with regard to Activin and Wnt converge toward a negative effect of the resulting increased Hedgehog signaling at both the early- and mid-stages of pancreas development. As we have recently shown, this negative effect of Hedgehog affects β-cell differentiation *per se*: mouse embryonic pancreas explants cultured in the presence of conditioned medium from embryoid bodies (EBs) (containing about 20 pg/ml of natural Shh protein) have a reduced number of insulin-positive cells, and β-cell differentiation can be rescued up to control levels by antagonizing the Hedgehog pathway alone (Mfopou et al., 2007).

A striking observation about Hedgehog in the pancreas was the identification of its signaling components in developing pancreatic tissue after E13.5 and in adult ducts and islets. Indeed, islet cells express the membrane proteins Smo and Ptc1, and the ligands Ihh and Dhh, suggesting a role for basal Hedgehog activity (Hebrok et al., 2000; Thomas et al., 2000; Kawahira et al., 2003; Kayed et al., 2003; Lau et al., 2006). The exact function of Ihh expressed in developing pancreas is not yet known. In contrast to increased pancreas/body ratio expected and observed in Shh −/−, double transgenic mice (Ihh −/−) display a proportional reduction in pancreas mass and body size, suggesting that Ihh contributes to organ growth (Hebrok et al., 2000). The function of Ihh in the pancreas deserves to be better investigated. Adult male *Ptc1*+/− transgenic mice, although normoglycemic after overnight fasting, were shown to display abnormal glucose tolerance, which suggests that Ptc1 activity might be required for proper islet function (Hebrok et al., 2000). These findings were further emphasized by the demonstration that recombinant Shh-N dose dependently induces *Pdx1* promoter activity and *Insulin* expression in INS1 and MIN6 cell lines, a process that was sensitive to Cyclopamine treatment (Thomas et al., 2000, 2001). While these studies clearly point to a role for Hedgehog signaling in adult islet, they do not establish a molecular link between this signaling and glucose homeostasis. For instance, increasing Hedgehog signaling in islets/cell lines by Ptc1 heterozygous mutation or by treatment with Shh-N results in two situations that are apparently contradictory: impaired glucose tolerance and increased Pdx1/insulin expression, respectively. Furthermore, there are still some gaps to fill as far as Hedgehog function in adult pancreas is concerned. For instance, *Shh* transcripts were not detected in adult pancreas in both studies (as well as in our unpublished data) and activation of *Pdx1* and *Insulin* transcription was demonstrated on cell lines. Although all Hedgehog ligands bind to the same receptor with similar affinity, it is clearly recognized that they elicit different intracellular responses depending on the context of their operation,

like cell fate specification, cell proliferation, cell survival, or the nature of target cell (Ingham and McMahon, 2001).

Hedgehog activity during ES cell differentiation

Early studies neglected normal pancreas developmental features and Hedgehog signaling

Major progresses made in islet transplantation in diabetic patients are facing an important hold back characterized by the shortage of islets, the basic ingredient of this therapeutic option. This situation fostered investigations aiming at establishing relevant alternatives to cadaveric islets, which in the past few years resulted in the publication of several papers describing ES cell potential to generate progenies that share some characteristics with the insulin-producing β-cell. These included Pdx1/insulin expression, glucose sensing, insulin secretion, and normalization of blood glucose in diabetic animals (Kahn, 2004; Weir, 2004). We screened the literature using the keywords embryonic stem, pancreas, and insulin and identified 50 original studies dealing with differentiation of insulin-producing β-like cells from ES cells. More than half of these studies (54%) were published in the last 2 years, indicating a growing interest in this field. Interestingly a large majority do not explicitly describe derivation of definitive endoderm progenitor cells as the first step used to generate β-cells, although in the study by Shiraki et al. (2005), endoderm differentiation was improved by *Cmix* overexpression whereas in a few others, Activin treatment was applied. Only very recently, *in vitro* differentiation of human ES cells into C-peptide+ insulin-producing cells was demonstrated via a protocol that involved sequential exposure of cells to extracellular factors mimicking pancreas developmental signals and allowing progressive transitions from definitive endoderm, primitive gut tube, posterior foregut, pancreatic endoderm, endocrine precursor to hormone-producing cells (D'Amour et al., 2006). In the same line, no more than four of those studies investigated the expression of Hedgehog components by differentiating ES cells; and only three studies evaluated the effects of Hedgehog antagonism with anti-Shh antibody or Cyclopamine (Blyszczuk et al., 2003; Leon-Quinto et al., 2004; Rolletschek et al., 2004; Skoudy et al., 2004; Mfopou et al., 2005; Shiraki et al., 2005; D'Amour et al., 2006). It therefore appears that many studies that claimed to generate insulin-producing cells actually did not follow the developmental pathway that is recognized for these cells. For instance, nestin-positive cell selection used by many authors might enrich in neuroectoderm progenitor cells, a lineage known to express the non-pancreas specific Insulin II gene in mice. Furthermore, this process has been shown to be associated with apoptosis and insulin uptake from the medium in which this hormone is supplemented up to 25 μg/ml (Lumelsky et al., 2001; Hori et al., 2002; Blyszczuk et al., 2003; Moritoh et al., 2003; Rajagopal et al., 2003; Hansson et al., 2004; Segev et al., 2004; Sipione et al., 2004; Bai et al., 2005; Paek et al., 2005). It is also known that insulin staining can be found in EBs generated from HNF6-null ES cells cultured without insulin supplementation, reflecting expression of this protein by the visceral endoderm, which in contrast to pancreas does not require the HNF6-Ngn3 cascade (Houard et al., 2003). All these suggest one should be cautious while interpreting data related to pancreas differentiation from ES cell studies, and that efforts should be made for *in vitro* mimicking of molecular events that govern pancreas development, starting with the establishment of a definitive endoderm lineage.

Hedgehog pathway components are enriched in differentiating ES cells

During development, Hedgehog pathway plays a major role in the patterning of several organs. Using EC and ES cells as model systems, expression of *Ihh* has been identified on the outer visceral endoderm layer and demonstrated to be involved in extraembryonic endoderm differentiation, yolk sac angiogenesis, and neuroectodermal differentiation (Becker et al., 1997; Grabel et al., 1998; Maye et al., 2000, 2004; Byrd et al., 2002). Similarly, expression of *Shh* was shown to be up-regulated upon ES cell differentiation, and exogenous Shh improves generation of neuronal cell types (Lee et al., 2000; Blyszczuk et al., 2003; Mizuseki et al., 2003; Li et al., 2005b; Mfopou et al., 2005). We recently showed that the active N-terminal fragment of Shh protein is secreted in the medium by cultured EBs, and potently activates downstream targets *Ptc1* and *Gli1* in cultured embryonic liver cells and in E12.5 mice pancreas explants. The medium from EBs remarkably altered embryonic pancreas differentiation *ex vivo*, resulting in the severe loss of epithelial tissue and dilation of ducts (Mfopou et al., 2007), all reminiscent of the features found in *Pdx1-Shh* or *Pdx1-Ihh* transgenic mice (Kawahira et al., 2005). These data infer that the biological microenvironment created by differentiating EBs might not be compatible with pancreatic fate, therefore explaining the limitations known so far in the field of β-cell derivation from ES cells. Considering the early requirement for Shh exclusion in the endoderm before pancreas initiation, this pathway might be regarded as the earliest barrier to the differentiation of pancreatic lineages from ES-derived endoderm cells (Mfopou and Bouwens, 2005; Mfopou et al., 2005).

Endoderm induction and pancreas initiation from ES cells: a role for Hedgehog signals?

It is now widely accepted that strategies to derive β-cells from ES cells should recapitulate developmental features of the pancreas (Fig. 1; Loebel et al., 2003; Stoffel et al., 2004; Bonner-Weir and Weir, 2005). It is thus essential to setup a definitive endoderm phenotype at the initial stages of differentiation. It is worth noticing that the identification of this cell population *in vitro* remains difficult to achieve in a straightforward fashion because there exists no marker unique to definitive endoderm and not shared with visceral endoderm or other germ layers. A combination of markers is therefore recommended. Several studies in *Xenopus* and mouse implicated transforming growth factor β (TGFβ) signaling in definitive endoderm induction, a process that goes via establishment of common mesendoderm progenitor cell expressing Gsc and Bry (Zorn et al., 1999; Kimelman and Griffin, 2000; Xanthos et al., 2001; Tam et al., 2003). Application of this knowledge to mouse and human ES cell lines resulted in the efficient derivation of definitive endoderm cells (25%–80%) expressing *Gsc*, *Sox17*, *Foxa2*, *Mixl1*, and *CxCR4* (Fig. 1). Those cells could be terminally differentiated into hepatocytes, pneumocytes, and enterocytes upon implantation in mice (Kubo et al., 2004; D'Amour et al., 2005; Pfendler et al., 2005; Shiraki et al., 2005; Tada et al., 2005; Yasunaga et al., 2005).

We questioned why pancreatic cells did not arise from ES-derived definitive endoderm cells after implantation in mice or after further differentiation *in vitro*. Considering our inhibitory microenvironment hypothesis (Mfopou et al., 2005), we evaluated expression of Hedgehog components upon endoderm induction by EBs culture in serum free medium with or without Activin A supplementation. Indeed, this procedure is highly and consistently associated with up-regulation of Hedgehog ligands, receptors, and effectors, resulting in about five-fold down-regulation of the key pancreas transcription factor *Pdx1* compared with control EBs maintained in 10% FBS (Fig. 3A–3C). Shh concentrations measured by ELISA in conditioned media ranged from 400 to 1,200 pg/ml; the protein could not be detected from fresh culture media (Fig. 3D–3E). These concentrations (see "The enigmas of Hedgehog processing, function, and synergizing factors") are within the range known to inhibit pancreas differentiation *in vitro* (Deutsch et al., 2001; Mfopou et al., 2007). The increase in Hedgehog components noticed after 7 days of Activin treatment might be a result of endoderm induction as this germ layer extensively expresses Hedgehog ligands, which are induced by Tcf2 (also known as Hnf1β) (Jensen, 2004). In this scope, our data are not contradictory to those of Nakanishi et al. (2007) who showed reduced Shh expression in EBs treated with lower concentrations of Activin A and Retinoic acid between culture days 4 and 6.

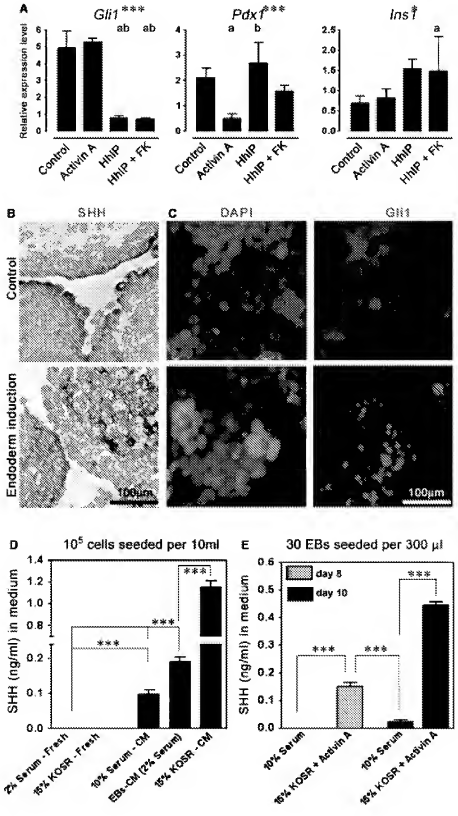
Antagonizing Hedgehog activity during ES cell differentiation

In the past, very few studies dealing with pancreas differentiation considered a role for Hedgehog ligands produced by EBs or tried blockade of this pathway as is required *in vivo*. Two authors used anti-Shh antibody (5E1, 2 μg/ml) and Cyclopamine (0.1 and 20 μM), to block the pathway in culture models not optimal for definitive endoderm differentiation, but did not confirm their effectiveness by analysis of downstream targets *Gli1*, *Ptc1*, and *Hhip*. Nevertheless, they showed improved *Pdx1* and *Insulin* expression upon Hedgehog antagonism, though higher Cyclopamine concentration decreased *Insulin* levels, a phenomenon suggestive of toxicity (Leon-Quinto et al., 2004; Skoudy et al., 2004).

As we noticed an increased Hedgehog signaling during definitive endoderm induction, and a subsequent down-regulation of *Pdx1* expression in EBs, we assessed the efficiency and effects of Hedgehog antagonism by use of several inhibitors including anti-Shh antibody, cyclopamine, HhIP, and Forskolin. It appeared that only very high concentrations of HhIP could reduce target gene expression by more than 70% with a concomitant five-fold up-regulation of *Pdx1* transcription (Fig. 3A; Mfopou et al., 2007). These data suggested that *Pdx1* down-regulation in Activin A-treated cells is Hedgehog dependent, and that Hedgehog activity generated by endoderm induction is very potent and requires high concentrations of soluble inhibitors for its antagonism. This was supported by a recent study by

Fig. 3 Hedgehog signals in differentiating embryonic stem cells and effects on pancreas differentiation. (**A**) On day 10 of endoderm induction (Activin A), expression of the Hedgehog target *Gli1* is blocked by Hedgehog interacting protein (HhIP, 10 μg/ml) alone or combined with Forskolin (FK, 20 mM). On day 6, *Gli1* transcripts were three-fold higher in Activin A condition than in control (not shown). *Pdx1* is down-regulated in the context of endoderm induction (Activin A), and this phenomenon is significantly blocked by addition of Hedgehog antagonists, resulting in increased *Insulin 1* levels. (**B–C**) Up-regulation of Hedgehog components Shh (B) and Gli1 (C) on day 10 of endoderm induction. Note that Shh staining is confined to the outer layer of visceral endoderm in control embryoid bodies (EBs). (**D–E**) ELISA for Shh in conditioned media (CM) from EBs cultured in serum (10% or 2% fetal bovine serum), in serum free medium (SFM) (15% knock-out serum replacement, KOSR), or in SFM+Activin A. Endoderm induction is associated with higher levels of secreted Shh. $^{*}p<0.05$ by analysis of variance (ANOVA); $^{***}p<0.001$ by ANOVA; a, $p<0.05$ versus Control; b, $p<0.05$ versus Activin A.

D'Amour et al. (2006), showing that hESC induced by Activin A give rise to insulin+ cells only when Hedgehog pathway has been inhibited early after definitive endoderm formation. Similarly, another study in mouse ES cells differentiated into EBs and treated for 2 days with a combination of Activin A and all-trans retinoid



acid showed activation of pancreatic endocrine and exocrine genes in a context of Shh repression (Nakanishi et al., 2007). It is therefore apparent that as occurs *in vivo*, Hedgehog pathway represents the earliest known pancreas inhibitor that should be repressed for pancreatic cells to develop from ES-derived definitive endoderm cells.

The enigmas of Hedgehog processing, function, and synergizing factors

The C-terminal end of Hedgehog molecules contain proteolytic activity, allowing their auto-processing, which generates a 19 kDa N-terminal active fragment and a 27 kDa C-terminal fragment devoid of signaling activity (Bumcrot et al., 1995; Porter et al., 1995). Following cleavage, the C-terminus of the active fragment is modified by cholesterol attachment while the N-terminus undergoes palmitoylation, myristoylation, stearolylation, or arachidoylation. These lipid modifications are required for Hedgehog to function as a morphogen, for multimeric formation, and for a 15- to 160-fold amplification of its potency (Pepinsky et al., 1998; Taylor et al., 2001; Zeng et al., 2001; Chen et al., 2004; Feng et al., 2004). They also explain why wild-type Shh is detected at low concentrations in culture supernatant even after cell transfection with vectors encoding Shh, as well as the large difference in the amount of recombinant Shh-N needed to elicit similar effects as those from naturally processed Shh (Bumcrot et al., 1995; Zeng et al., 2001).

Using conditioned medium from EBs to culture mouse E12.5 pancreas explants or embryonic liver cells, we found that an average Shh concentration of 200 pg/ml elicited *Ptc1* and *Gli1* inductions five and 15 folds higher than recombinant Shh-N peptide used at 2.5 μg/ml. Furthermore, attempts to rescue pancreas explant growth and differentiation by HhIP supplementation were unsuccessful in undiluted conditioned medium (200 pg/ml Shh), whereas β-cell differentiation was rescued in 10% diluted conditioned medium after antagonizing Hedgehog activity alone (Mfopou et al., 2007). This suggests that wild-type Hedgehog ligands present in the conditioned medium were very potent as expected, though other synergizing pathways such as Wnt and FGF might play additional or synergistic roles (Hart et al., 2003; Hebrok, 2003; Mfopou et al., 2005; Heiser et al., 2006). Indeed, it was recently shown that concomitant activation of the PI3K/Akt pathway significantly improves Shh-induced target gene expression and that Wnt activates Gli1 transcription through modulation of Gli2/3 (Borycki et al., 2000; Riobo et al., 2006).

Another enigma to deal with is the differential expression of Hedgehog ligands and targets in early and late pancreas epithelium on one hand, and the non-specificity of available Hedgehog antagonists on the other hand. In other words, it appears that in contrast to Shh, Ihh is expressed in the pancreas at later stages of development and in the adult, and might contribute to the normal morphogenesis of this organ. If the expression of this particular ligand at a certain stage of pancreas derivation from ES cells is required or mandatory, solving this issue would be made difficult by the fact that available Hedgehog antagonists are not absolutely ligand specific, therefore limiting the ability to tightly control Ihh-dependent/Shh-independent target gene activity *in vitro* (Hebrok et al., 2000; Thomas et al., 2000; Hebrok, 2003; Kawahira et al., 2003; Lau et al., 2006).

Concluding remarks and perspectives

The initial fever of deriving insulin-producing cells from ES cell by shortcuts is now waning. The position of the definitive endoderm as mandatory precursor cells is well recognized and substantial progress has been achieved in deriving such cells by modulation of TGFβ signaling. Further differentiation of ES-derived definitive endoderm toward pancreatic cells by modulation of known pancreas regulatory pathways has also been recently achieved, giving rise to 7% insulin+ cells. The transition from definitive endoderm to pancreatic epithelium is controlled and restricted *in vivo* and *in vitro* by Hedgehog signaling, a pathway that is highly activated in ES-derived endoderm cells. Its inhibition appears mandatory especially at early stages for the generation of pancreatic cells from ES cells, but the requirement of a certain threshold of Hedgehog signal from Ihh and Dhh for pancreas morphogenesis might complicate the procedure as selective antagonists are not available. A combination of strategies is to be considered, for instance pan-Hedgehog inhibition at early stages (KAAD-Cyclopamine, Forskolin, HhIP, RNA interference for *Gli* effectors, *Ptc1/2* and *Hhip* overexpression), selective Hedgehog inhibition at later stages (RNA interference for *Shh* only), together with supplementation of soluble factors implicated in pancreatic development or maintenance (Activin B, Ffg2, Glp1, Exendin4, Betacellulin, Hgf, Retinoid acid, Nicotinamide) and adequate control of the selection of endocrine versus exocrine fate by Notch inhibitors. While awaiting the refinement of the best protocol known so far (D'Amour et al., 2006; Madsen and Serup, 2006), definitive endoderm induction from transgenic ES cell lines with mutations in Hedgehog components might provide additional and substantial information on the influence of this pathway on pancreas fate initiation from ES-derived endoderm cells.

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